

**Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol**

**Supplemental Assay Method for the Evaluation of Koch's
Old Tuberculin**

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Supplemental Assay Method for the Evaluation of Koch's Old Tuberculin

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Supplemental Assay Method for the Evaluation of Koch's Old Tuberculin

1. Introduction

This is a Supplemental Assay Method (SAM) procedure for the evaluation of production lots of Koch's old tuberculin in accordance with the Code of Federal Regulations, Title 9 (9 CFR), Part 113.406.

2. Materials

2.1 Reagents/supplies

2.1.1 *Mycobacterium tuberculosis* reference tuberculin, current lot. This reference is obtained from the United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Veterinary Services (VS), Center for Veterinary Biologics-Laboratory (CVB-L).

2.1.2 *M. tuberculosis* sensitizing agent, current lot. This reagent is available from the CVB-L.

2.1.3 Saline solution, 0.85%, pH 6.4

2.1.4 Metric ruler made of clear plastic

2.1.5 Needles, 20 ga x 1 in and 26 ga x $\frac{3}{8}$ in

2.1.6 Disposable syringes, 1 ml and 3 ml

2.1.7 Pipettes, 5 ml and 25 ml

2.1.8 Glass serum bottles, 10 ml and 125 ml

2.1.9 Rubber seals and metal caps for serum bottles

2.1.10 Crimper for aluminum seals

2.1.11 Depilatory cream, NairTM or equivalent

2.1.12 Animal clippers

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2.2 Animals

2.2.1 Guinea pigs, 500-700 g, white-haired, nonpregnant females. Ten guinea pigs are required for each lot to be tested. Two additional guinea pigs are required as nonsensitized controls. All guinea pigs used for a test must be from the same source and housed and fed in the same manner.

3. Preparation for the test

3.1 Personnel qualifications/training

Technical personnel must have working knowledge of the use of general laboratory chemicals, equipment, and glassware and have specific training and experience in the safe handling of laboratory animals.

3.2 Selection and handling of test animals

3.2.1 Select guinea pigs that are healthy, free of external parasites, and have an unblemished hair coat.

3.2.2 Examine guinea pigs the day they are received, and house according to the current version of the National Veterinary Services Laboratories (NVSL)/CVB-L Animal Users' Manual.

3.2.3 When the test is concluded, instruct the animal caretakers to euthanize and incinerate the guinea pigs and to sanitize the cages according to the current version of ARSSOP0004.

3.3 Preparation of reagents

3.3.1 Saline solution, 0.85% (NVSL Media 30201)

Sodium chloride	8.5 g
Water	q.s. 1.0 L

Adjust pH to 6.4.
Autoclave at 121°C for 15 min.

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3.4 Preparation of supplies

3.4.1 Sterilize all glassware before use.

3.4.2 Use only sterile supplies (syringes, needles, rubber seals, metal caps, etc.).

3.5 Test animal sensitization

3.5.1 Sensitize 10 guinea pigs per lot of tuberculin to be evaluated. Wait **30 to 120 days** before performing the potency portion of the assay.

3.5.2 Administer 0.5 ml of *M. tuberculosis* sensitizing agent intramuscularly to each guinea pig. Split the dose, administering 0.25 ml into each rear leg. Use 3-ml syringes fitted with 20-ga x 1-in needles.

3.5.3 Retain 2 guinea pigs as nonsensitized controls.

4. Performance of the potency test

4.1 Preparation of guinea pigs for the potency assay

Clip the abdomen of each guinea pig with animal clippers. Generously apply a depilatory cream to the clipped abdomen. Wait at least 10 min. Wash off the depilatory cream with warm water and dry the abdomen with a soft towel. Allow the guinea pigs to rest for 3-4 hr before administering the tuberculin injections.

4.2 Preparation of tuberculin dilutions

4.2.1 Preparation of control dilutions

1. Shake bottle several times to mix. Remove 0.5 ml of reference tuberculin from the bottle using a 1-ml tuberculin syringe. Add tuberculin to a 10-ml serum bottle containing 1.5 ml saline, to create a 1:4 dilution. Cap the bottle, label, and mix well by shaking.

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2. Add 1 ml of the 1:4 dilution of tuberculin, measured in a 1-ml tuberculin syringe, to a 10-ml serum bottle containing 1.5 ml of saline to create a 1:10 dilution. Cap the bottle, label, and mix well by shaking.
3. Repeat **Sections 1. and 2.** with the lot of tuberculin to be tested.

4.2.2 Preparation of assay dilutions

Note: Prepare dilutions of the reference tuberculin and the lot to be tested in exactly the same manner.

1. Dispense 99 ml of saline into a 125-ml serum bottle. Add 1.0 ml of reference tuberculin, measured in a 1-ml tuberculin syringe. This is a 1:100 dilution. Cap and label the bottle. Mix well by shaking.
2. Label 2, 10-ml serum bottles 1:200 and 1:400, respectively. Use a 5-ml pipette to add 5.0 ml of saline to each of the bottles labeled 1:200 and 1:400.
3. Use a 5-ml pipette to add 5.0 ml of the 1:100 dilution prepared in **Section 1.** into the bottle labeled 1:200. Rinse the pipette once by filling the pipette and expelling the solution. Discard this pipette. Close the bottle with a rubber stopper. Mix well by shaking.
4. Use a 5-ml pipette to transfer 5.0 ml of the 1:200 dilution into the bottle labeled 1:400. Rinse the pipette as described above. Close the bottle with a rubber stopper. Mix well by shaking.
5. Clamp aluminum seals over the rubber stoppers on the 3 serum bottles containing the diluted tuberculin.

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6. Repeat **Sections 1.-5.** with each lot of tuberculin to be tested.

4.3 Intradermal injection of test animals

4.3.1 Identify 6 injection sites on the abdomen of each sensitized guinea pig, with 3 sites on each side, evenly spaced, equidistant from the midline. Record this information on the worksheet. (See sample worksheet in **Section 9.1.**) Do not mark the sites on the abdomen with ink.

4.3.2 Each sensitized guinea pig will receive a total of 6 injections, 1 at each previously identified site. Randomly assign which preparation (1:100, 1:200, or 1:400 dilution of the reference tuberculin; or 1:100, 1:200, or 1:400 dilution of the test tuberculin) will be injected into each site. Record this information on the worksheet.

4.3.3 Administer 0.05 ml of each preparation at the sites determined in **Section 4.4.2** by intradermal injection. Use 1-ml tuberculin syringes fitted with 26-ga x $\frac{3}{8}$ -in needles.

4.3.4 Identify 4 injection sites on each nonsensitized (control) guinea pig, equidistant, 2 on each side of the midline. Record this information on the worksheet. Do not mark the sites on the abdomen with ink.

4.3.5 Each nonsensitized (control) guinea pig will receive a total of 4 injections, 1 at each previously identified site. Randomly assign which preparation (1:4 or 1:10 dilution of the reference tuberculin; or 1:4 or 1:10 dilution of the test tuberculin) will be injected into each site. Record this information on the worksheet.

4.3.6 Administer 0.05 ml of each preparation at the sites determined in **Section 4.4.5** by intradermal injection. Use 1-ml tuberculin syringes fitted with 26-ga x $\frac{3}{8}$ -in needles.

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5. Interpretation of the test results

5.1 Recording of test results

5.1.1 Measure the test reactions at 23-25 hr following injection.

5.1.2 Measure the greater and lesser diameters of erythema and/or swelling to the closest mm at each injection site (see **Figure 1**). Gently palpate the lesion to determine the margin of the swelling, which may or may not extend beyond the margin of erythema. Record the results on the test record (**Section 9.2**).

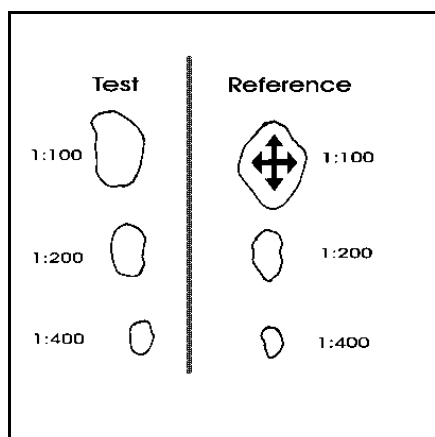


Figure 1.

5.1.3 Calculate the area of erythema and/or swelling (in mm²) by multiplying the greater and lesser diameter measurements.

5.1.4 For each dilution of tuberculin tested, add together the reaction areas for that dilution from each of the 10 guinea pigs. Record the sum reaction for each dilution on the test record.

5.1.5 Add together the sums obtained for each dilution of the reference tuberculin to generate the grand total for the reference tuberculin. Similarly calculate a grand total for the test serial. Record these results on the test record.

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5.1.6 Divide the grand total for the test serial by the grand total for the reference tuberculin. Multiply by 100 to express the test serial reaction as a percentage of the reference tuberculin reaction. Record all calculations on the test record.

5.2 Criteria for a valid test

5.2.1 The control guinea pigs must have no signs of reaction at 24 hr.

5.2.2 For a satisfactory serial, the reaction of the test serial must be $\pm 25\%$ of the reaction of the reference tuberculin, i.e., the percentage calculated in **Section 5.1.6** must fall between 75% and 125%.

6. Reporting of test results

6.1 Report test results as described by BBSOP0020, current version.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.406, U.S. Government Printing Office, Washington, DC, 1998.

7.2 History of reagents: The *M. tuberculosis* sensitizing agent is a moist, heat-killed preparation of *M. tuberculosis* strains Pn, C, and DT. These strains were originally obtained from Cooper Animal Health. *M. tuberculosis* reference tuberculin was obtained from the USDA, APHIS, VS, NVSL, Ames, Iowa 50010.

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8. Summary of revisions

This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. No significant changes were made from the previous protocol.

9. Appendices

9.1 Site injection worksheet

9.2 Test record

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9.1 Site injection worksheet

Old Tuberculin Injection Site Identification

Sample Code _____

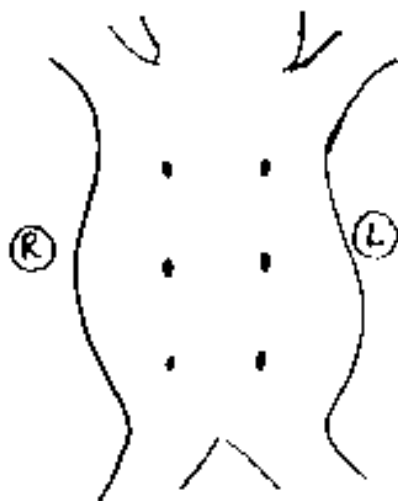
Date _____

Test Guinea Pigs:

ID _____

ID _____

ID _____



ID _____

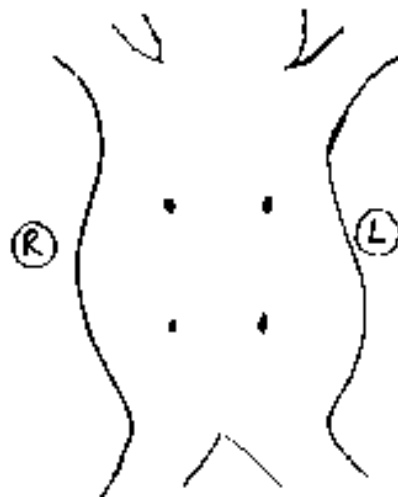
ID _____

ID _____

Control Guinea Pigs

ID _____

ID _____



ID _____

ID _____

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9.2 Test record

Test Record
Guinea Pig Potency Test, Old Tuberculin

Est. number	Product Code	Serial Number	Sample Code
		Lot Number	
Sensitigen			
Reference Tuberculin			
Depilatory			

Animal Information	
Animal Order Number	
Animal Room Number	
Caretaker	
Average weight of 5 randomly selected guinea pigs at sensitization	

	Date/Time	Initials
Sensitized		
Depilatory applied		
OT injected		
Reactions read		

Results ☐ Satisfactory ☐ Unsatisfactory ☐ No test ☐ Inconclusive

Comments:

Date

Signature

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Appendix 9.2 Test record continued

Guinea Pig No.	Reactions to Reference Tuberculin (length x width, in mm)					
	1:100 reference	mm ²	1:200 reference	mm ²	1:400 reference	mm ²
1	x		x		x	
2	x		x		x	
3	x		x		x	
4	x		x		x	
5	x		x		x	
6	x		x		x	
7	x		x		x	
8	x		x		x	
9	x		x		x	
10	x		x		x	
Total						
Grand Total						

Guinea Pig No.	Reactions to Test Serial (length x width, in mm)					
	1:100 test serial	mm ²	1:200 test serial	mm ²	1:400 test serial	mm ²
1	x		x		x	
2	x		x		x	
3	x		x		x	
4	x		x		x	
5	x		x		x	
6	x		x		x	
7	x		x		x	
8	x		x		x	
9	x		x		x	
10	x		x		x	
Total						
Grand Total						

Percent response=(Grand total test serial÷Grand total reference tuberculin)x100= _____%

Controls				
Guinea Pig No.	Reference Dilution	Reaction (in mm)	Test Serial Dilution	Reaction (in mm)
1	1:4	x	1:4	x
	1:10	x	1:10	x
2	1:4	x	1:4	x
	1:10	x	1:10	x